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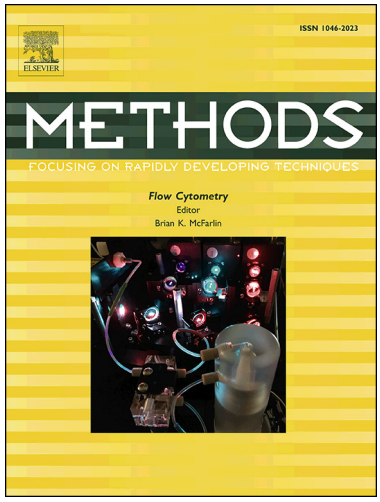

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In vitro production of synthetic viral RNAs and their delivery into mammalian cells and the application of viral RNAs in the study of innate interferon responses.

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Keywords: RNA, virus, dendritic cells (DCs), interferon (IFN) response, *in vitro* RNA production, RNA delivery, DsiRNA, innate immunity

Abbreviations:

DC: dendritic cell

DdRp: DNA-dependent RNA polymerase

DsiRNA: Dicer-substrate siRNA

HPLC: high-performance liquid chromatography

IFN: interferon

moDC: monocyte-derived dendritic cell

PRR: pattern recognition receptors

RdRp: RNA-dependent RNA polymerase

RLR: RIG-I-like receptor

sfRNA: subgenomic flavivirus RNA

shRNA: short hairpin RNA

siRNA: small interfering RNA

TLR: Toll-like receptor

Highlights:

1. Different ssRNA and dsRNA can be produced *in vitro* using bacteriophage T7 DNA-dependent RNA polymerase and phi6 RNA-dependent RNA polymerase, respectively.
2. RNA can be delivered into cells by various transfection methods, however, the timing, amount, purification and the reagents for RNA transfection should be carefully considered.
3. Produced ssRNA and dsRNA can be applied e.g. in studying host innate immune responses, demonstrating that RIG-I preferentially recognizes short dsRNAs/ssRNAs bearing 5'end triphosphate group, while MDA5 recognizes longer dsRNA molecules.
4. This *in vitro* RNA synthesis system can produce DsiRNA swarms with basically any virus (or microbe) specificity for protecting host cells from virus infection.

Summary (words 238)

Mammalian cells express different types of RNA molecules that can be classified as protein coding RNAs (mRNA) and non-coding RNAs (ncRNAs) the latter of which have housekeeping and regulatory functions in cells. Cellular RNAs are not recognized by cellular pattern recognition receptors (PRRs) and innate immunity is not activated. RNA viruses encode and express RNA molecules that usually differ from cell-specific RNAs and they include for instance 5'capped and 5'mono- and triphosphorylated RNAs, small viral RNAs and viral RNA-protein complexes called vRNPs. These molecules are recognized by certain members of Toll-like receptor (TLR) and RIG-I-like receptor (RLR) families leading to activation of innate immune responses and the production of antiviral cytokines, such as type I and type III interferons (IFNs). Virus-specific ssRNA and dsRNA molecules that mimic the viral genomic RNAs or their replication intermediates can efficiently be produced by bacteriophage T7 DNA-dependent RNA polymerase and bacteriophage phi6 RNA-dependent RNA polymerase, respectively. These molecules can then be delivered into mammalian cells and the mechanisms of activation of innate immune responses can be studied. In addition, synthetic viral dsRNAs can be processed to small interfering RNAs (siRNAs) by a Dicer enzyme to produce a swarm of antiviral siRNAs. Here we describe the biology of RNAs, their *in vitro* production and delivery into mammalian cells as well as how these molecules can be used to inhibit virus replication and to study the mechanisms of activation of the innate immune system.

1. Introduction

Ribonucleic acids (RNAs) are versatile macromolecules which exist in many different sizes and forms and they possess various essential biological functions. The fundamental function of RNAs is to mediate genetic information from DNA to proteins as well as to regulate important steps in the cellular life cycle. In the 1950s and 1960s, RNA was first known to participate in protein biosynthesis and three types of RNAs in eukaryotic cells were found, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) [1-4]. During the following six or seven decades, additional RNA types and their different forms and modifications were identified. It is evident that different types of housekeeping and regulatory RNA molecules have essential and complicated functions in the life cycle of cells and whole organisms. RNA viruses express various types of RNA molecules which are often structurally different from host RNAs and they are thus recognized as foreign structures by different cellular receptors, which leads to the activation of host innate immune responses.

2. RNA classification

Endogenous RNAs can be classified into two groups: coding RNAs and non-coding RNAs (ncRNAs) (Fig. 1). Coding RNAs, also known as mRNAs are transcribed from DNA and they are translated into polymers of amino acids to form proteins. In eukaryotic cells gene transcription leads to the production of precursor mRNAs (pre-mRNAs) which are further processed into mature mRNAs via extensive RNA

processing steps (splicing). NcRNAs are not translated into proteins. However, there are many types of ncRNAs which play different vital roles during the cellular life cycle. For example, as housekeeping ncRNAs, rRNAs are the key components of ribosomes, the protein synthesis machinery of the cell [5-7]; tRNAs participate in mRNA translation by carrying amino acids to ribosomes and by acting as adaptor and decoder molecules to translate the codon sequence of mRNA to amino acid sequence in proteins [8-10]; small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) molecules, found in the nucleus of eukaryotic cells, are involved in post-transcriptional modification of RNAs [11]; snRNA is responsible for alternative splicing of pre-mRNA molecules to define sequences to be translated [12]; and snoRNA is involved in rRNA and tRNA modification, mRNA editing and genome imprinting [13]. Moreover, there are several regulatory ncRNAs which can be divided into long ncRNAs and short ncRNAs according to the length of RNAs. Long ncRNAs include e.g. circular intronic and circular RNAs (ciRNA, circRNA), long intergenic ncRNA (lincRNA), and pseudogene transcripts. They act as important regulators of diverse biological processes [14-16]. Short ncRNAs are comprised of microRNAs (miRNA) [17], piwi-interacting RNAs (piRNA) [18] and small interfering RNAs (siRNA) [19], which are involved in RNA silencing and post-transcriptional regulation of gene expression via different mechanisms and pathways [20]. In order to prevent cellular RNA from 5’ to 3’ exonuclease cleavage and triggering innate immune responses, the 5’end of endogenous RNAs are usually protected by various structural elements. For instance, the 5’end of mRNA is blocked by a cap structure - a

5’-5’ linked N7-methyl guanosine (m7G) cap to the first nucleotide of the RNA [21]. The protection of non-coding RNAs is normally achieved by forming a ribonucleoparticle with its associated proteins which shield 5’ end of RNAs [22]. Moreover, some lncRNAs such as circRNAs do not harbor open 5’ and 3’ ends since they form a ring structure and link the 3’ and 5’ ends with a back splicing covalent bond [23].

Beside endogenous RNAs, different types of foreign RNAs can be released while cells are invaded by various pathogens such as viruses (Fig. 1) [24]. These RNAs include viral genomic ssRNAs and dsRNAs, RNA replication intermediates with different 5’end groups, small viral RNAs generated during infection [25], and special RNA-protein complexes, such as vRNPs formed during influenza virus infection [26]. Unlike in endogenous RNAs, the 5’end structure of most foreign RNA molecules are often exposed. Viral genomic ssRNAs, dsRNA intermediates and some small aberrant viral RNA transcripts bearing 5’end triphosphate groups can be recognized by a group of microbial sensors called pattern recognition receptors (PPRs), which then trigger innate immune responses by activating multiple host cell signaling pathways [27]. Disturbances in this surveillance system may lead to inflammatory or autoimmune diseases. Although the host has intricate recognition systems to discriminate between microbial and endogenous RNAs [28], misprocessed, or mislocalized host-derived RNAs generated during virus infection can also be recognized by PRRs, such as Retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs) and Toll-like receptors (TLRs), leading to activation of abnormal innate immune responses [29]. However,

during the evolution some viruses have developed various mechanisms to modify their genomic and mRNA 5' ends. Such structures include e.g. an RNA cap which addition is catalyzed by specific viral capping enzymes or is obtained by cap-snatching, i.e. by stealing the cap from host mRNA [21]. Many ssRNA viruses also have a short protein tag, known as viral protein genome-linked or VPg, covalently bond to the 5'end of the genomic ssRNA. Alternatively, viruses may shield the 5'end of RNA via non-covalent interactions with proteins as in vRNP viral polymerase complex structure [26] or by enclosing the viral RNA in protein or lipid compartments which protect the RNA. These mechanisms allow efficient transcription of viral RNAs, avoiding viral RNA degradation by cellular 5'–3' exonucleases, and circumventing the viral RNA recognition by the host cell.

3. *In vitro* production methods for RNA molecules

During the past decades RNA-related research has made significant progress and the demand for different RNAs has increased. The development of methods to produce different types of RNAs or RNA mimics *in vitro* has been essential for various fields of research contributing to our understanding on the role of various cellular RNAs, the recognition of invading pathogenic RNAs, and RNA-induced activation of host innate immune responses. Besides synthesizing polyinosine–polycytidylic acid (poly I:C) as a dsRNA analog [30], presently two main approaches are used to produce RNAs *in vitro*: chemical synthesis and enzymatic production. **The chemical synthesis method** for RNA production is normally implemented as an automated solid-phase synthesis

159 from ribonucleoside phosphoramidite substrates [31]. However, the need to protect
160 the 2'-hydroxyl group of the substrate molecules during the chemical synthesis of
161 RNA creates steric hindrance which greatly reduces the coupling efficiency of RNA
162 phosphoramidite monomers and thus the RNA yields [32]. Moreover, this approach
163 mainly generates ssRNA molecules and for the production of dsRNAs there is a need
164 to hybridize the cognate ssRNAs. The chemical synthesis method has also other
165 limitations: the method is relatively inaccurate in producing RNA molecules with a
166 correct length and sequence. It is also expensive which restricts its industrial or
167 clinical application possibilities, and the size-range of produced RNA is small,
168 typically less than 100 nucleotides, while naturally expressed, biologically active
169 RNAs are generally substantially longer [33].

170 The classical way of **enzymatic RNA generation** is *in vitro* transcription of
171 target DNA sequence containing the T7 polymerase promoter utilizing T7
172 DNA-dependent RNA polymerase (DdRp) [34]. With this method long ssRNA
173 molecules with basically any sequence specificity can be produced, which is a clear
174 benefit compared to chemical synthesis of short RNAs. However, in order to produce
175 dsRNAs, DNA templates in both sense and antisense orientation should be designed
176 and the produced ssRNA transcripts must be hybridized after *in vitro* production. The
177 accuracy of hybridization is often compromised, especially in the case of long ssRNA
178 molecules, due to the formation of ssRNA secondary structures by self-annealing or
179 false-annealing between the strands. Therefore, the yield of produced dsRNA is often
180 limited since many mis-annealed dsRNA or biologically inactive dsRNA molecules

are generated during the annealing step.

In order to avoid the defects and limitations of the traditional dsRNA production methods, our laboratory has introduced an enzymatic dsRNA synthesis platform, making the enzymatic production of different size and type of RNAs possible (Fig. 2, Supplementary Material). DNA target sequences are first designed according to the application of desired RNA products, and ssRNAs with different lengths (short, medium size or long) are *in vitro* transcribed by T7 DdRp. Unlike in the classical dsRNA production method, different dsRNAs can be directly synthesized from the produced ssRNA templates by bacteriophage phi6 RNA-dependent RNA polymerase (RdRp) [35]. The phi6 RdRp is produced as a recombinant protein, purified to homogeneity and used for *in vitro* synthesis of dsRNA. The enzyme has high processivity and low template specificity which enables efficient production of dsRNAs from a wide variety of heterologous ssRNA templates [35,36]. The generated ssRNAs or dsRNAs can subsequently be further modified, e.g. by dephosphorylation or capping, depending on the needs of further applications [37]. Generated ssRNAs and dsRNAs can be then precipitated by LiCl or ethanol precipitation, or be obtained by Trisol extraction method, and be desalted by a NAP column system and further purified by HPLC purification before their use in various chemical or biological processes [37].

Moreover, the developed RNA synthesis platform also allows us to produce a target-specific siRNA swarm to be used as antiviral substances against viral infections which may provide an efficient and potential novel method for inhibiting emerging

viral infection outbreaks (Fig. 2, Supplementary Material). Designed target DNA sequences can be used to directly generate desired dsRNAs in a single-tube process combining T7 DdRp catalyzed ssRNA transcription and phi6 RdRp-based dsRNA synthesis. DsRNAs can then be further digested by *Giardia intestinalis* or human Dicer enzymes to generate the swarms of Dicer-substrate siRNA (DsiRNA) [39,42], followed by purification through anion-exchange chromatography on a monolithic QA column [38,39] or GEN-PAK FAX column (Supplementary Material and [36]) and NAP column desalting [38,39]. DsiRNA swarms generated *in vitro* can target multiple viral genomic sequences and mRNAs so that infection of viruses even with high genetic variation (e.g. influenza virus, hepatitis C virus, human immunodeficiency virus) can be efficiently inhibited by the DsiRNA swarm as compared to a single-target siRNA approach [40]. Moreover, low concentration of each individual siRNA type in the swarm reduces the possibility of severe off-target effects [41]. Indeed, several studies have demonstrated that DsiRNA swarms generated with this novel method can efficiently inhibit both DNA and RNA virus infections without inducing detectable off-target effects [36,39,42-45].

For example, our recent research [42] showed that an *in vitro* synthesized influenza A virus-specific siRNA efficiently inhibits the replication of several influenza A virus strains in human primary cells (Fig. 3), confirming that a swarm of virus-specific siRNA can act as a broad-spectrum inhibitor.

4. RNA delivery methods

During the past decades, researchers have been trying to overcome one of the major

challenges for utilizing *in vitro* synthesized RNAs in basic and applied research – the efficacy of intracellular delivery of RNA. RNAs, especially ssRNAs, are prone to degradation by endogenous RNA-degrading enzymes. Moreover, due to their relatively large molecular size and strong negative charge, RNAs cannot readily cross negatively charged cellular membranes [46]. Proper RNA delivery methods are required to improve RNA entry into the cells and to avoid endosomal uptake in order to get RNAs transported directly into the cell cytoplasm [47]. Currently, various means of RNA delivery have been developed. Generally, RNA delivery can be mediated by viral and non-viral carriers. Adenoviruses [48], adeno-associated viruses [49], lentiviruses [50,51] and retroviruses [52] are commonly used viral carriers for RNA delivery. However, all these viral carriers are mainly used for the delivery of short RNA molecules, such as siRNAs and short hairpin RNAs (shRNAs) [53]. Although these vectors are suitable for the delivery of siRNAs into almost all types of cells, and can be applied into *in vivo* studies due to their very high transduction efficacy and long-term silencing efficacy, their applicability is still limited due to concerns in biosafety, such as risks of high immunogenicity and potential insertional mutagenesis associated with the application of viral vector in RNA delivery [53]. To circumvent safety concerns and other limitations of viral vectors, non-viral delivery systems, which comprise cationic polymers and lipids, have been extensively developed as well [54]. Although these carriers have lower efficiency as compared to their viral counterparts, they are easy to produce and safer to use, and they provide the possibility to deliver a broader range of RNA types. As nanoparticle-based delivery

247 vehicles, lipid-based carriers and natural polymers including chitosan, atelocollagen,
248 cationic polypeptides, and synthetic polymers, such as linear and branched
249 polyethyleneimine (PEI), polyL-lysine (PLL), linear polyamido amines (PAA) and
250 polyamido amine dendrimers (PAMAM), have been widely used for RNA delivery
251 [55]. Of these, the lipid-based carriers are the most studied ones and commonly used
252 for intracellular RNA delivery [56]. Cationic lipids can form bilayers due to their
253 amphipathic properties and they can electrostatically bind negatively charged RNA
254 molecules to form lipid-RNA complexes which have the natural capacity to merge
255 with cellular membranes to mediate RNA uptake into cells [53,54,57]. However, we
256 know that some types of cells, such as human primary cells, are difficult to transfect.
257 Therefore, novel transfection methods and reagents are urgently required. Currently,
258 several novel transfection methods have been developed and used for RNA delivery,
259 including electroporation, sonoporation and magnetofection. Electroporation is a
260 physical transfection method which creates temporarily small pores in cell membranes
261 by applying an electrical field to increase the permeability of cell membranes to
262 facilitate the entry of RNAs into cells [58]. This technique has very high transfection
263 efficiency and has been widely applied in the transfection of all kinds of cells
264 including human primary cells such as human dendritic cells and neurons [59,60].
265 Similar to electroporation where RNA is driven into cells by an electrical force along
266 the electric field, sonoporation is a transfection method using ultrasound to form small
267 pores in cell membranes leading to transfer of RNA into the cells [61]. In
268 sonoporation RNA delivery is mediated by passive diffusion and the method is less

toxic than RNA transfection methods with viral carriers. However, the efficiency depends on ultrasound frequency and intensity, and similar to electroporation, it may damage target cells. Magnetofection is a simple and highly efficient transfection method which utilizes metallic nanoparticles coated with cationic molecules to form complexes with nucleic acids by electrostatic and hydrophobic interactions [62]. The particles containing RNAs are then concentrated and transported into the target cells by an appropriate magnetic field. This method combines the advantages of biochemical and physical transfection methods reducing the disadvantages of single methods (low efficiency, high toxicity). Magnetofection enables cells to be RNA transfected without physically damaging the cells.

5. RNA application in the research of innate immunity

Recent years have witnessed an explosion in interest and research in innate immunity. Pathogen recognition by PRRs is an early and essential step in triggering the innate immune system [24]. How PRRs recognize different pathogen-associated molecular patterns (PAMPs) and how they discriminate self from foreign RNA molecules [27,28] are currently extensively studied questions in the field. Detailed analyses of such phenomena rely on the use of specifically designed RNA mimics. With our versatile RNA synthesis platform, we can generate various types of pathogen-specific RNA mimics, such as mimics of short or long viral ssRNA or dsRNA molecules generated during virus infection, viral genomic RNAs with 5'end triphosphate groups, and mimics of self RNAs with 5'end cap structure (Fig. 2). These RNA molecules can be

applied to stimulate different types of host cells or experimental animal models in order to better study the mechanisms of activation of the innate immune system. The potential of siRNAs as antiviral substances can also be studied [36,39,42-45].

As the sentinel cell of the host, the human dendritic cell (DC) is one of the key cell types regulating innate and adaptive immunity. It functions as a major antigen-presenting cell linking innate and adaptive immune systems [63,64]. Several PRRs are expressed in DCs which recognize different PAMPs upon the invasion of microbial pathogens [65]. Innate immune responses in early stages of infection in DCs can be specifically studied using PAMP mimics, such as different types of *in vitro*-produced RNA molecules. However, as primary cells, DCs are very fragile upon stimulation and efficient delivery of RNAs into the cells is demanding. We compared the RNA transfection efficiency of several commercially available transfection reagents in DCs and analyzed their effects on cell viability. Although most of the transfection reagents possessed relatively high RNA transfection efficiency, some of them led to increased cell death, especially when used with high amounts of RNA (Fig. 4). Moreover, according to our previous study, transfection of siRNAs against influenza A virus should take place before virus infection in order to get host cells well protected against the infection [42]. It is noteworthy that all *in vitro* enzymatically produced RNAs should be purified by a NAP column desalting and anion-exchange HPLC before RNA delivery, otherwise the stability and transfection efficacy of transfected RNAs and the cell viability after RNA delivery will be dramatically reduced. Therefore, the timing, amount, purification and the reagents for

313 RNA transfection should be carefully chosen.

314 Several PRRs, which reside in host cells, recognize different types of foreign
315 RNAs and trigger the induction of type I and III interferons (IFNs) through multiple
316 intracellular signaling pathways [66,67]. RNA-recognizing PRRs include RLRs and
317 TLRs. RLRs reside in the cell cytoplasm, such as the RIG-I molecule which mainly
318 recognizes short RNAs bearing a 5'end triphosphate group, and melanoma
319 differentiation-associated gene 5 (MDA5) which preferentially recognizes long
320 dsRNAs [68]. In humans, the TLR family includes altogether 10 members of which
321 TLR3, TLR7 and TLR8 are RNA-recognizing receptors. These TLR molecules are
322 expressed on the plasma membrane (TLR3) or in endosomes and the endoplasmic
323 reticulum (TLR3, TLR7 and TLR8) [69]. Currently, chemically synthesized RNAs or
324 RNA analogues are the most used agonists to study RNA recognition by RLRs or
325 TLRs [69-75]. Initially Dr. Kato and his colleague identified the length-dependent
326 recognition by RIG-I and MDA5 by using different-size poly I:C as an RNA agonist
327 [76,77]. Poly I:C is synthesized by annealing different size single-stranded inosine
328 poly(I) and cytidine poly(C) homopolymers resulting in a mixture of dsRNA
329 analogues which have a wide size range (such as low and high molecular weight poly
330 I:C in size ranges of 0.2kb-1kb and 1.5kb-8kb, respectively). In addition to the size
331 distribution, poly I:C lacks any sequence specificity for microbial (viral) genomes or
332 RNAs. Furthermore, homopolymeric sequences do not exist in nature and inosine is a
333 minor component in natural RNAs, questioning the relevance of poly I:C as an RNA
334 mimic. With our enzymatic RNA production platform, we can synthesize ssRNAs and

dsRNAs (short, medium size and long RNAs) ranging from 58bp up to 3kb [37] with substantially higher sequence accuracy than by using chemical synthesis from any microbial sequence. Previous studies using poly I:C suggested that RIG-I preferentially recognizes RNA molecules of less than 1kb and MDA5 recognizes dsRNA molecules larger than 1kb [76]. Our result from RIG-I and MDA5 knock-out (KO) and RIG-I/MDA5 double KO cells stimulated with enzymatically produced dsRNAs showed that RIG-I can still recognize 1.8 kb-long dsRNAs and MDA5 can recognize as short as 300 bp long dsRNAs (Fig. 5). By using *in vitro*-produced, different size, sequence and modification-specific RNAs we can get more detailed information e.g. of the length-dependent recognition by RLRs. Moreover, by stimulating human primary macrophages and DCs with *in vitro* enzymatically synthesized ssRNAs or dsRNAs with different length and different 5'end group, we noticed that RIG-I plays a dominant role in recognizing foreign, non-host-specific RNAs in human primary cells [37]. So with the novel *in vitro* RNA synthesis method, we can obtain a versatile collection of RNA mimics in order to study in more detail what type of RNAs are recognized by different PRRs. We can also further study the mechanisms how PRRs distinguish foreign and endogenous RNAs.

Besides *in vitro*-synthesized RNAs as foreign RNA mimics, it is also important to study how natural viral RNAs activate (or suppress) innate immune responses during viral infections. It is well known that several types of viral RNAs that are generated during virus infection are capable of inducing innate immune responses via different signaling pathways. These RNA molecules include viral genomic RNAs, viral RNA

intermediates and small viral RNAs generated during virus transcription and replication [25,66,68,78-81]. Specifically, small viral RNAs generated during virus infection have an essential role in viral life cycle. Several studies have demonstrated that during flavivirus, such as West Nile virus (WNV) infection, an abundant pool of small RNAs, with a size distribution of 0.3 to 0.5 kb, is produced. These noncoding subgenomic flavivirus RNAs (sfRNAs) are derived from the 3' untranslated region of the viral genome [82,83] and have several important functions during flavivirus infection [84], such as facilitating viral replication, viral pathogenicity [85], and viral evasion from type I interferon-mediated antiviral responses [86]. Furthermore, by using deep sequencing, Dr. Velthuis and his colleagues identified mini viral RNAs with the size range of 56–125 nt generated during influenza A virus infection, which can efficiently trigger IFN responses via the RIG-I pathway [87]. This result is consistent with our data showing that *in vitro*-synthesized small ssRNA and dsRNA molecules, with the length from 58 nt to 128 nt, have a better efficacy to induce IFN responses via the RIG-I pathway than longer RNAs [37].

Moreover, some viral RNA-protein complexes, such as vRNPs of influenza viruses, can also activate innate immune responses [26]. Therefore, it is very useful to separate different types of natural viral RNAs or produce viral RNA mimics to further study the mechanisms of innate immune responses activated during viral infections. For example, we produced or isolated several types of influenza viral RNAs, including total viral RNAs extracted from virus stock of influenza A virus, *in vitro*-synthesized genomic viral RNAs specific for each of the eight influenza A virus

segments, pooled viral genomic RNAs, and RNAs extracted from virus infected cells, and compared the ability of these RNAs to induce innate immune responses [88]. We found that most of these viral RNAs can induce IFN responses with similar efficiency, which indicates that the sequence of influenza viral RNA is not the key factor regulating the induction of innate immune responses (Fig. 6). Instead, RNA length and the structural modifications are essential in the ability of viral RNA to induce innate immunity. These factors include e.g. small viral RNAs generated during virus replication [89] and 5'end triphosphate groups of genomic viral RNAs [37]. Since the replication strategy of different virus groups may be very different and also the RNA structures produced in different viral infections may vary, it is extremely important to identify the RNA variety for different viral pathogens. This information will give us better understanding of the pathogenesis of different viral infections and will likely provide us opportunities for the development of RNA-based antiviral therapies.

6. Concluding remarks

In conclusion, viral RNAs and their structural variables have become more and more important for the study of host innate immune responses and as potential targets for RNA-based therapeutics against viral infections. Therefore, methods for producing, separating and identifying different types of viral RNAs are urgently needed. Besides methods described above to produce various viral RNAs or their RNA mimics, researchers have also started to use other methods to separate and identify extracted viral RNAs. These methods include e.g. RNA purification with steric exclusion

chromatography [90] and RNA deep sequencing methodology which allows identification of different viral genomic and small RNAs [91,92]. For instance, Dr. Russell and his colleague set up a new approach by using single-cell virus sequencing to determine the sequences in single cells infected by viruses [93]. However, the development of efficient methods to identify the variety of RNA structures is still in its early stage. But when more and more novel methods are applied for identifying different RNA structures, we will definitely have a broader view of the mechanism how foreign RNA is recognized and discriminated by the host innate immune system.

7. Ethics statement.

Adult human blood was obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Transfusion Service (renewed annually).

8. Authorship

M.J., P.Ö., M.M.P. and I.J. conceived the study, M.J. wrote manuscript draft, M.J., P.Ö. M.M.P. and I.J. co-revised manuscript.

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664

665 **Figure legends**

666

667 Figure 1. RNA classification. RNAs can be divided into endogenous RNAs and
668 foreign RNAs such as RNAs delivered or synthesized during viral infections in the
669 cells (dark green ellipse). Endogenous RNAs are transcribed from different DNA
670 sequences (orange and yellow chimeric rectangle) in the nucleus (dotted green ellipse)
671 and are classified into two groups: coding RNAs, which are first transcribed to
672 pre-mRNAs (orange and yellow chimeric serrated rectangle) and are then processed
673 into mRNAs (orange serrated rectangle); non-coding RNAs (ncRNAs, yellow serrated
674 rectangle), which are not translated into proteins and they can be divided into
675 housekeeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs include
676 transfer RNA (tRNA), ribosomal RNA, small nuclear RNA (snRNA) and small
677 nucleolar RNA (snoRNA). Regulatory ncRNAs consist of long ncRNAs and short
678 ncRNAs. Long ncRNAs contain e.g. circle RNA (ciRNA), long intergenic ncRNA
679 (lincRNA), and pseudogene transcripts. Short ncRNAs include micro RNA (miRNA),
680 piwi-interacting RNA (piRNA) and small interfering RNA (siRNA).

681 Different types of RNAs can be released into cells during viral infections, such as
682 viral genomic ssRNAs with 5'end monophosphate or triphosphate, cap structure or
683 protein tags, small viral RNA (small vRNA) and dsRNA intermediates during virus
684 replication, and specific viral RNA-protein structures for instance vRNPs found in
685 influenza viruses or influenza virus-infected cells.

686

Figure 2. Enzymatic generation of different types of RNAs. (A) Enzymatic generation of different ssRNA or dsRNA molecules with different structures. A designed DNA sequence (rose red rectangle) is generated by PCR using forward primer harboring the promoter sequence for T7 DNA-dependent RNA polymerase at the 5'end. T7 RNA transcription is then performed to yield ssRNA (dark blue wavy lines) with different lengths (short ssRNAs <200nt, medium size ssRNAs 200−1000nt, and long ssRNAs >1000nt) using various DNA sequences as templates (top panel). A set of dsRNAs with the same lengths (light blue wavy lines) is generated from the ssRNA template by primer-independent phi6 RdRp-catalyzed RNA replication. Synthesized ssRNA and dsRNA molecules can be modified by cIAP enzyme to remove 5'end triphosphate group, or RNA molecules can be further modified e.g. by RNA capping. (B) Enzymatic production of DsiRNA swarm. Several target DNA sequences are chosen and chimeric DNA template is designed, followed by T7 RNA transcription to produce ssRNA with a chimeric target sequence (orange wavy line). DsRNA is generated from ssRNA template by primer-independent phi6 RdRp-catalyzed replication (light rose red wavy lines). The generated dsRNA is purified and cleaved by a Dicer enzyme to generate an siRNA swarm (green small wavy lines) containing different siRNAs targeting multiple RNA regions. Digestion with human Dicer produces canonical 21-nt long siRNA molecules while *Giardia intestinalis* Dicer can be used to produced 27-nt long siRNA. After introduction into human cell the 27-nt long siRNA is further processed by the endogenous Dicer and therefore such siRNA is called as Dicer-substrate siRNA (DsiRNA).

709

710 Figure 3. Broad-spectrum inhibition of influenza A virus replication in human moDCs
711 by pre-transfection with an influenza A virus-specific DsiRNA swarm. Human
712 moDCs from four different blood donors were separately pre-transfected with control
713 or negative siRNAs (neg siRNA) or eGFP DsiRNA or specific DsiRNA swarm
714 against influenza A virus (cIAV DsiRNA) (10 nM) for 21h. Cells were subsequently
715 infected by indicated influenza A virus strains (H5N1, H7N9, H3N2 and H1N1) at
716 multiplicity of infection 1. A. 24 h p.i. cells from different blood donors were pooled
717 and the M1 RNA expression of influenza A virus was determined by quantitative
718 reverse transcription PCR (qRT-PCR) from isolated total cellular RNA samples. The
719 values were normalized against β-ACTIN gene–specific mRNA, and relative M1
720 RNA levels of influenza A virus were calculated by the ΔΔCt method using untreated
721 cells as a calibrator. The means (±SD) of 3 parallel analyses are shown. Data are
722 representative of 3 individual experiments. The statistical significance is indicated as
723 (*) $P<0.05$ or (**) $P<0.01$ against samples of non-siRNA transfected cells (boxed
724 bars). Viral M1 RNA expression (top) is shown in relation to the immunoblot analysis
725 of viral PB1, NP, M1, NS1 proteins in siRNA/DsiRNA transfected moDCs infected
726 with the indicated strains of influenza A viruses. Human β-ACTIN protein levels were
727 analyzed as a control. Cells were collected at 24 h p.i., and whole cell lysates were
728 prepared. Cellular proteins (30 μg/lane) were separated by electrophoresis in 10%
729 SDS-polyacryl amide gel, followed by Western blot analysis with the indicated
730 antibodies. One representative experiment of three independent experiments is shown.

731 The figure is adapted from a figure in reference 43.

732

733 Figure 4. RNA transfection efficacy in primary human monocyte-derived dendritic
734 cells (moDCs) and cell viability of moDCs after RNA transfection. Human primary
735 monocytes obtained from four different blood donors were differentiated into moDCs
736 in 12-well plates. Then cells were un-transfected (control) or separately transfected
737 with BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (50nM or 25nM) using
738 indicated transfection reagents, including lipofectamine 3000 (LF3000), siPORT,
739 lipofectamine RNAiMAX (LF RNAiMAX), TransIT-siQuest (siQuest), TransIT-TKO
740 (TKO) and DharmaFECT1 for 24h. Cells were washed with 0.5% bovine serum
741 albumin in phosphate-buffered saline and collected and stained with propidium iodide
742 (PI) for cell viability analysis after transfection. The samples were analyzed with a
743 FACSCanto II (BD) device using FACSDiva software to analyse transfection efficacy
744 as Alexa Fluor expression and the rate of cell death as PI positivity.

745

746 Figure 5. Activation of innate immune responses with different-sized RNA molecules.
747 Mouse wild-type (wt) cells (expressing RIG-I and MDA5), RIG-I knock-out (KO)
748 cells (RIG-I -/-, lack of expression of RIG-I), MDA5 KO cells (MDA5 -/-, lack of
749 expression of MDA5), and RIG-I/MDA5 double-KO cells (RIG-I -/-, MDA5 -/-
750 double KO, lack of expression of both RIG-I and MDA5) (in 12-well plates; 5 × 105
751 cells/well) were mock transfected (control) or transfected with *in vitro*-produced
752 different size ssRNAs or dsRNAs (100 ng/ml). After 24 h of incubation, cells were

753 collected for RNA isolation and quantitative RT-PCR analysis. The values of
754 RT-PCR analyses were normalized against β-actin gene-specific mRNA, and the
755 relative Ifn-β mRNA level was calculated by the ΔΔCT method using untreated
756 control cells as a calibrator.

757

758 Figure 6. Analysis of activation of innate immune responses in human moDCs by
759 synthetic and natural influenza A virus-specific RNAs. MoDCs from four different
760 blood donors were mock transfected (control) or separately transfected with indicated
761 RNAs (100 ng/ml), including low molecular weight poly I:C, a synthetic analog of
762 dsRNA, viral RNA extracted from influenza A virus stock (vRNA), enzymatically
763 produced ssRNAs specific for each of the 8 genomic segments of influenza A virus
764 (ssRNA1 to ssRNA8), pooled ssRNAs (from ssRNA1 to ssRNA8), and RNAs
765 extracted from influenza A virus-infected cells. Influenza A virus-specific RNAs were
766 produced as previously described [88]. After 24 h of incubation, cells were collected
767 for RNA isolation. Relative expression of type III interferon, IFN-λ1 mRNA, was
768 measured by quantitative RT-PCR. IFN-λ1 mRNA values were normalized against
769 18S rRNA, and relative expression levels were calculated with the ΔΔCt method
770 using untreated cells as a calibrator.

771

772 Highlights:

5. Different ssRNA and dsRNA can be produced *in vitro* using bacteriophage T7 DNA-dependent RNA polymerase and phi6 RNA-dependent RNA polymerase, respectively.
6. RNA can be delivered into cells by various transfection methods, however, the timing, amount, purification and the reagents for RNA transfection should be carefully considered.
7. Produced ssRNA and dsRNA can be applied e.g. in studying host innate immune responses, demonstrating that RIG-I preferentially recognizes short dsRNAs/ssRNAs bearing 5'end triphosphate group, while MDA5 recognizes longer dsRNA molecules.
8. This *in vitro* RNA synthesis system can produce DsiRNA swarms with basically any virus (or microbe) specificity for protecting host cells from virus infection.